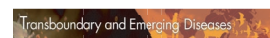


ORIGINAL ARTICLE



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Predicting the presence and titre of rabies virus-neutralizing antibodies from low-volume serum samples in low-containment facilities

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Abstract

Serology is a core component of the surveillance and management of viral zoonoses. Virus neutralization tests are a gold standard serological diagnostic, but requirements for large volumes of serum and high biosafety containment can limit widespread use. Here, focusing on *Rabies lyssavirus*, a globally important zoonosis, we developed a pseudotype micro-neutralization rapid fluorescent focus inhibition test (pmRFFIT) that overcomes these limitations. Specifically, we adapted an existing micro-neutralization test to use a green fluorescent protein-tagged murine leukaemia virus pseudotype in lieu of pathogenic rabies virus, reducing the need for specialized reagents for antigen detection and enabling use in low-containment laboratories. We further used statistical models to generate rapid, quantitative predictions of the probability and titre of rabies virus-neutralizing antibodies from microscopic imaging of neutralization outcomes. Using 47 serum samples from domestic dogs with neutralizing antibody titres estimated using the fluorescent antibody virus neutralization test (FAVN), pmRFFIT showed moderate sensitivity (78.79%) and high specificity (84.62%). Despite small conflicts, titre predictions were correlated across tests repeated on different dates both for dog samples ($r = 0.93$) and in a second data set of sera from wild common vampire bats ($r = 0.72$, $N = 41$), indicating repeatability. Our test uses a starting volume of 3.5 μ l of serum, estimates titres from a single dilution of serum rather than requiring multiple dilutions and end point titration, and may be adapted to target neutralizing antibodies against alternative lyssavirus species. The pmRFFIT enables high-throughput detection of rabies virus-neutralizing antibodies in low-biocontainment settings and is suited to studies in wild or captive animals where large serum volumes cannot be obtained.

KEYWORDS

biologic assay, *Desmodus rotundus*, generalized linear mixed models, immunofluorescence

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1 | INTRODUCTION

The last few decades have seen a surge in newly emerging human viruses that originate from wildlife (Cunningham et al., 2017; Daszak et al., 2000; Goodin et al., 2018). Key examples include Nipah virus (Gurley et al., 2017), Seoul virus (Kerins et al., 2018), 2009 H1N1 (Mena et al., 2016) and the recent SARS-CoV-2 (Zhou et al., 2020). Understanding the epidemiological dynamics of such viruses within their natural host populations is a fundamental component to discerning the spatiotemporal dynamics of past outbreaks and anticipating future emergence (Cunningham et al., 2017; Plowright et al., 2016). Investigating the dynamics of zoonotic viruses within wildlife presents multiple challenges such as limited sample sizes, biased sampling and multiple diagnostic tests that are difficult to compare. Moreover, viruses themselves may be undetectable at the moment of sampling when infectious periods are short or virus shedding is intermittent (Becker et al., 2019; Gilbert et al., 2013; Plowright et al., 2019). Consequently, key parameters needed to inform population-level disease dynamics (e.g., incidence, infection and incubation periods) are difficult or impossible to measure directly (Borremans et al., 2016). Serological tests offer a powerful alternative to approaches that rely on pathogen detection (Gilbert et al., 2013). Since pathogen-specific antibodies generally persist longer than the pathogen itself, serological data can inform individual exposure histories and population seroprevalence and, in some cases, help approximate the force of infection (Borremans et al., 2016; Gamble et al., 2020; Gilbert et al., 2013; Metcalf et al., 2016). However, applying serology to longitudinal studies of wildlife presents distinct challenges from tests used in clinical diagnostic settings, where accuracy takes precedence over scalability. In particular, serological tests for studies of wildlife should be amenable to the small volumes of serum that often characterize collections from small-bodied hosts (e.g., rodents, bats, birds), scalable to large numbers of samples required for such studies, and possible to implement in low-biocontainment laboratories.

Rabies lyssavirus (RV; Genus *Lyssavirus*, Family *Rhabdoviridae*) is a zoonotic virus transmitted in saliva by the bite of infected mammals (mainly Carnivora and Chiroptera) (Rupprecht et al., 2017). RV constitutes a substantial global health problem that is responsible for over 59,000 human fatalities annually, mostly attributable to domestic dogs (WHO, 2017). In Latin America, vampire bat RV causes more cases of human and domestic animal rabies mortality than RV transmitted by dogs. Similarly, bats are consistently among the most frequent sources of human rabies exposure in the United States (Ma et al., 2020; Schneider et al., 2009). Bat lyssaviruses exemplify the challenges of studying zoonoses using pathogen detection and the potential advantages of serological inference (Turmelle et al., 2010). Active infections are rarely detected due to low population-level incidence and the short infectious period that precedes death (Jackson et al., 2008; Turmelle et al., 2010). However, abortive infections (i.e., bats that are exposed to RV but do not become infectious) are routinely observed through antibody presence in apparently healthy bats (Constantine et al., 1968; Jackson et al., 2008;

Obregón-Morales et al., 2017; Steece & Altenbach, 1989). As such, antibody detection is commonly used for ecological and epidemiological studies of bat rabies (Blackwood et al., 2013; Costa et al., 2013; de Thoisy et al., 2016; George et al., 2011; Horton et al., 2020; Streicker et al., 2012; Wright et al., 2010).

Existing tests to detect RV antibodies include enzyme-linked immunosorbent assays (ELISAs) (Barton & Campbell, 1988; Cliquet et al., 2004; Wasniewski & Cliquet, 2012) and virus neutralization tests (Cliquet et al., 1998; Smith et al., 1973). ELISAs are a rapid and effective method for testing large numbers of samples, but they do not detect virus neutralization (Ma et al., 2012; Welch et al., 2009). Moreover, since ELISAs ideally require a secondary antibody specific to the host species of the original sample (Reynes et al., 2004), which is often non-existent, they are not widely used in wildlife rabies surveillance. Tests detecting virus-neutralizing antibodies (VNAs) are more commonly adopted (De Benedictis et al., 2012; Irie & Kawai, 2002; Moore & Hanlon, 2010). Specifically, the fluorescent antibody virus neutralization test (FAVN) and the rapid fluorescent focus inhibition test (RFFIT) are considered the gold standard for measuring vaccination response by the World Health Organization (WHO) (Briggs et al., 1998; De Benedictis et al., 2012). Both the FAVN and RFFIT have been modified to facilitate use with wildlife samples. For instance, Kuzmin et al. (2008) modified the RFFIT into a micro-neutralization test using 4-well Teflon-coated slides instead of 8-well chamber slides. This produced a sensitive and specific test that only required 3.5 µl of serum (regular RFFIT and FAVN require ~50 µl of serum) (Kuzmin et al., 2008). Several other laboratories have introduced the modification of pseudotype viruses (i.e., non-rabies viruses engineered to express heterologous envelope glycoproteins) to quantify VNA titres without highly pathogenic live lyssaviruses (Temperton et al., 2015), which in most countries involves biosafety-level (BSL)-3 laboratories to produce concentrated virus stocks (WHO, 2018). Pseudotyped viruses based on vesicular stomatitis virus (Moeschler et al., 2016), human immunodeficiency virus and murine leukaemia virus (MLV) (Wright et al., 2008) tend to be highly sensitive and specific relative to live lyssavirus counterparts. Viral pseudotypes have also been modified to incorporate molecular biomarkers such as firefly luciferase, renilla luciferase or green fluorescent protein (GFP), eliminating the need to fix and stain cells with a fluorescein isothiocyanate (FITC)-conjugated rabies antibody, which reduces test cost (Bentley et al., 2015; Mather et al., 2013; Moeschler et al., 2016; Moore & Hanlon, 2010; Wright et al., 2008).

Despite these improvements, existing variations of RV neutralization tests have several limitations. First, all tests require serial dilutions of serum samples, each of which requires manual microscope scoring that increases personnel time and risks of error (Moeschler et al., 2016; Péharpré et al., 1999). Second, testing is commonly split into screening and end point titration phases, requiring tests to be run in duplicate or sequentially. Third, pseudotypes have not yet been integrated into micro-neutralization tests, implying that tests to detect VNAs against most lyssaviruses in their natural bat reservoirs can only be carried out in BSL-3 or higher facilities. The growing demand to study wildlife on large spatial and temporal scales

would benefit from minimizing these logistical constraints (Becker et al., 2019).

The ideal serological diagnostic would be a scalable virus neutralization test that requires low-volume samples and can be carried out in any standard microbiology/cell culture laboratory. Here, the micro-neutralization RFFIT is adapted to use an MLV-based viral pseudotype bearing the RV glycoprotein and carrying a GFP marker gene. Further, we introduce a novel quantitative approach that combines digital image analysis of infected cells with statistical analysis, which allows us to estimate rabies virus-neutralizing antibody (RVNA) titres without multiple serum dilutions or rounds of testing. Our approach relies on the expectation of a negative relationship between RVNA concentrations in serum and the number of cells that become infected upon viral challenge in the presence of that serum. Defining that relationship quantitatively using known titres of standard rabies immune globulin (SRIG) allows predicting the presence

and, when appropriate, titres of RVNAs in test sera. Our approach, which we refer to as a pseudotype micro-neutralization RFFIT (hereafter, pmRFFIT, Figure 1), provides a safe, low-cost alternative to standard RV neutralization tests that is suitable for large-scale, population-level studies or laboratory studies where small animals are longitudinally sampled.

2 | METHODS

2.1 | Laboratory work

2.1.1 | Cell lines

For all the neutralization tests, mouse neuroblastoma cells N2A (Neuro-2A, ATCC® CCL131™) were cultured in Minimum Essential

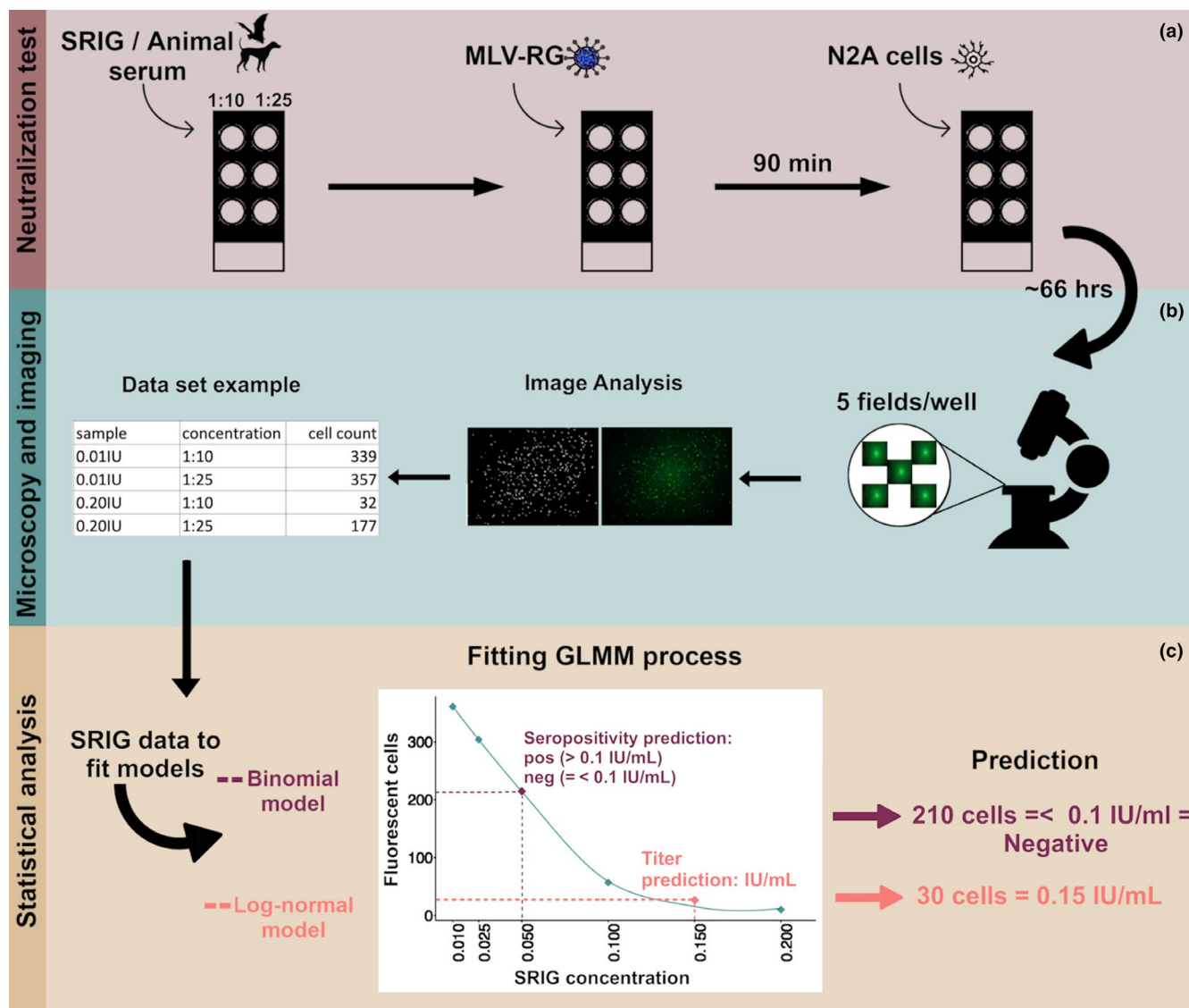


FIGURE 1 Workflow of the pmRFFIT approach. (a) Set-up of the neutralization test using an MLV(RG) pseudotype and 2 dilutions of either SRIG or animal serum. (b) Microscopy phase and imaging to perform the cell count of the fluorescent cells to construct a database to fit the statistical models. (c) Construction of the statistical models with two different types of prediction [Colour figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com)]

Media (MEM) supplemented with 10% foetal bovine serum (FBS), 1% 100× non-essential amino acid (NEAA), 1% 200mM L-glutamine and 1% antibiotic-antimycotic. For viral pseudotype production, human embryonic kidney 293T cells (293T, ATCC® CRL-3216™) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 1% 200 mM L-glutamine, 100 units/ml penicillin, 1 µg/ml streptomycin and 400 µg/ml G418 (Geneticin) antibiotic.

2.1.2 | Pseudotype production

MLV pseudotypes expressing the challenge virus standard-11 (CVS-11) rabies virus glycoprotein (MLV(RG)) were generated by co-transfection of pCMVi (MLV gag-pol expression vector), pCNCG (MLV viral origin and GFP reporter gene) and pl.18-CVS-11 (plasmid encoding the CVS-11 RG) (Bock et al., 2000; Towers et al., 2000; Wright et al., 2008). Plasmids were transfected using polyethyl- enimine (Polysciences, Inc.) into 293T cells at a ratio of 1:1.5:1 of pCMVi/pCNCG/pl.18-CVS-11. After 72 hr, the supernatant containing the viral pseudotypes was harvested, aliquoted and stored at -80°C until further use. Virus concentration was determined in N2A cells by calculating the TCID₅₀ (50% tissue culture infective dose) using the end point method and using the Spearman-Kärber formula (Condit, 2001; Hierholzer & Killington, 1996).

2.1.3 | Neutralization test

A two-dilution micro-neutralization test was established following Kuzmin et al. (2008); however, in place of 4-well Teflon-coated slides, 6-well Teflon-coated slides (Tekdon Inc.) were used, enabling 3 samples to be analysed per slide. Each serum sample (starting volume of 3.5 µl) was screened at a 1:10 and 1:25 dilution (a total of 2 wells per sample). Serum dilutions were inoculated with 12.5 µl of MLV(RG) (viral input: 300–400 TCID₅₀) and incubated in humidified square petri dishes at 37°C, 5% CO₂ for 90 min. After incubation, 25 µl of N2A cells (2 × 10⁶ cells/ml) was added to the serum-virus mixture and slides were incubated at 37°C, 5% CO₂ for 66 to 72 hr in the humidified square petri dishes. This extended incubation period (compared to the FAVN (48 hr) or the RFFIT (20 to 24 hr)) was required for the MLV(RG) to generate sufficient GFP expression for later microscopy. Since our pseudotype virus used GFP to indicate viral entry into cells, neither acetone fixation nor staining with anti-rabies monoclonal globulin was required. Every neutralization test included three internal controls: (1) a cell-only control where no viral pseudotype or serum was added to the well; (2) a viral pseudotype control, comprising back titrations of the MLV(RG) with (a) the original viral pseudotype concentration (300–400 TCID₅₀), (b) a 1:10 dilution and (c) a 1:100 dilution; and (3) a negative control for neutralization that included two wells with 300–400 TCID₅₀ of MLV(RG) without any serum. A standard curve was generated with each neutralization test describing the expected number of infected cells versus titre concentration. For this, the same protocol was used with

six different titre concentrations of SRIG in lieu of serum samples: 0.01, 0.025, 0.05, 0.1, 0.15 and 0.2 IU/ml (reconstituted at 30 IU/ml in 1.0 ml of nuclease-free water, 2nd WHO International Standard, Human; NIBSC, UK) (Figure 1a).

2.1.4 | Imaging and data processing

After incubation, slides were photographed at 4× magnification under a fluorescence microscope (EVOS FL Cell Imaging System). As representative images of the whole well, five equally sized fields (four corners and centre) were selected and photographed clockwise from the top left corner, such that the fifth photograph was always the centre field. Each photograph was processed through the freely available software, ImageJ (version 1.52k (Rasband, 2018)) using the Autolocal Threshold Phansalkar plugin (size radius 15) (part of the Fiji distribution (Schindelin et al., 2012)). Each image was transformed into a binary representation (every pixel stored as a single bit), indicating the presence (white) and absence (black in the background) of GFP fluorescence. Next, the command "Analyze Particles" was used to count the total number of fluorescent cells per field (i.e., infected cells). This command grouped and counted the white neighbouring pixels with a pre-determined size area and circularity to be a single cell (size area: 5–50 circularity: 0.80–1.0), so counts corresponded to the number of infected cells. Cell count outputs were converted into a standardized spreadsheet using a Python version 3.7.2 script (Python Core Team, 2019) (script available in supplementary information). At the end of the image processing step, each serum sample was described by 10 data points consisting of the number of the fluorescent cells in each of 5 fields (photographs) in the 1:10 and 1:25 dilutions (Figure 1b).

2.2 | Statistical analysis

All statistical analyses were executed in R (R Core Team, 2020). To predict the probability of RVNA presence, a generalized linear mixed model (GLMM) with a binomial distribution was fit to the binary outcome of the cell counts from the SRIG concentration series. Titres ≤ 0.1 IU/ml were considered RVNA-negative and > 0.1 IU/ml were considered RVNA-positive. Other serology studies in wildlife have used similar thresholds to detect RVNA (Araujo et al., 2014; Campos et al., 2019; Marcelo Azevedo de Paula Antunes et al., 2017; Silva et al., 2010). To predict RVNA titres, a GLMM with a log-normal distribution was fit to the infected cell counts across the SRIG concentration series. For both the binomial and log-normal models, a model was constructed including all data (i.e., from the 1:10 and 1:25 dilutions). For this model, two fixed effects were considered: (1) the count of virus-infected N2A cells (scaled to improve model convergence) and (2) the serum dilution level (two factors: 1:10 and 1:25 dilution). Random slope and intercept terms were considered for the date the test was run ("test date") to account for observed variation in the relationships between SRIG titres and infected cell counts

across dates (Figure 2). A random slope term was also considered for the field number (1 to 5) within each microscope well ("field") to account for variation in cell counts between fields (the middle field, field 5, had more agglomerated cells in particular). To evaluate whether a simpler, single dilution test produced comparable results, the full data set was then subset to the 1:10 dilution only. The binomial and log-normal models fit to this data subset included only the fixed effect of the virus-infected N2A cell counts, but the random effects were identical to those explained above (i.e., test date and field). Models were fit using the *lme4* package (Bates et al., 2015). The *predict* function was used to generate the predicted probability that a SRIG concentration or serum sample was seropositive (binomial model) and its corresponding RVNA titre (log-normal model). Predictions per field were averaged to obtain results per sample (Figure 1c).

2.2.1 | Model selection

To select the best-performing models, a top-down model selection strategy was implemented (Zuur et al., 2009). First, we identified the optimal random effects by comparing models with alternative random-effect structures, but the same fixed effects, using the Akaike information criterion corrected for small sample size (AICc). This was calculated using the *MuMIn* package (Bartoń, 2020). A lower AICc with a difference of at least two values ($\Delta AICc > 2$) was considered as evidence of improved model fit. Next, keeping the chosen random effects, models with the two different data sets were evaluated (simpler model: 1:10 data set, and more complex model: 1:10 and 1:25 data set) (Table 1). Since models fit to different data sets cannot be compared through AICc, the simpler and more complex models were compared using predictions for SRIG (sera with known

titres). For the binomial models, the sensitivity and specificity with a threshold of > 0.1 IU/ml for seropositivity were used. For the log-normal models, the Spearman correlation coefficients of predicted and known SRIG titres were compared.

2.2.2 | Validation of the pmRFFIT

To understand the variability of the pmRFFIT, replicate SRIG titre concentration curves were produced on 6 different dates between 30/05/2019 and 27/06/2019 (hereafter "Test 1" through "Test 6"). We evaluated dispersion in the counts (and hence potential predictive power) for the 1:10 versus the 1:25 dilution series of different SRIG concentrations by calculating the interquartile range (IQR) of the infected cell count.

Dog ($N = 47$) and bat ($N = 41$) sera were used to assess pmRFFIT repeatability. Dog sera had previously been tested using FAVN at the Animal Health and Veterinary Laboratories Agency, Weybridge (Wright et al., 2009). Bat sera were collected from common vampire bats (*Desmodus rotundus*) as part of an ongoing field study in Peru (collection permit: 0142-2015 SERFOR/DGGSPFFS; exportation permit: 003327-SERFOR). Specific and individual sample information is available in the supplementary information (SI, Table S1).

To quantify repeatability, dog and bat sera were processed using the pmRFFIT as described above on two separate dates. For the binomial model, repeatability was measured as the proportion of samples with the same predicted serological status on both test dates. For the log-normal model, repeatability was calculated as the Spearman correlation coefficient of predicted RVNA titres from different test dates.

We measured the accuracy of the pmRFFIT as the overall proportion of positive and negative samples correctly detected relative to

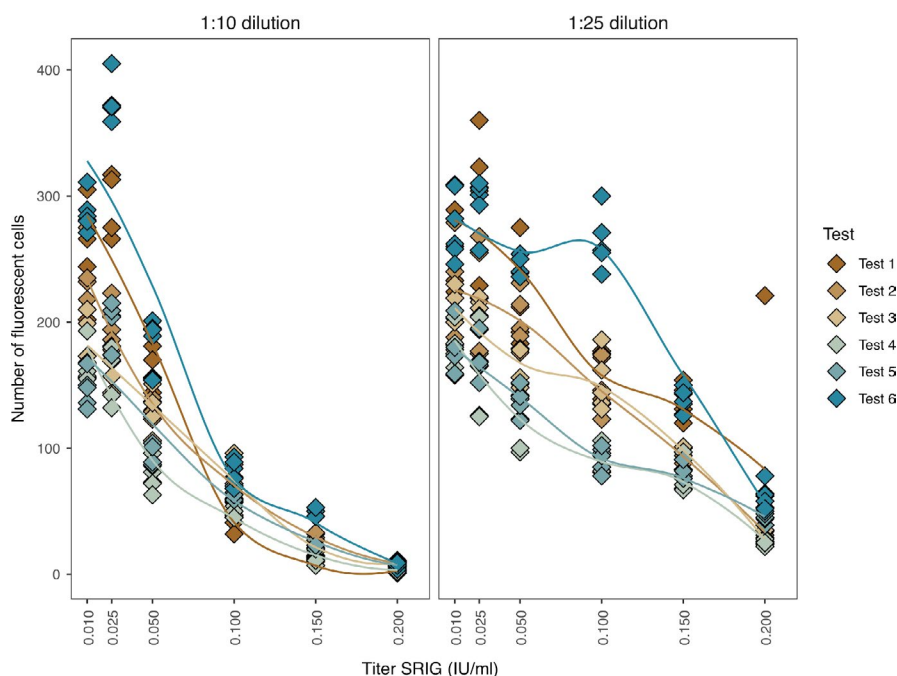


FIGURE 2 Raw counts of fluorescent cells for the SRIG concentration curves from 6 different pmRFFIT from different test dates and for each dilution. The dilutions were performed on the same date, with one well next to the other. Each colour represents a different test date. The loess curves denote differences in the counts from test to test [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Random-effect structures investigated for the binomial (B) and log-normal (L) models, and respective model fit measures: AICc, difference in AICc from best model (Δ AICc) and weight (W). The fixed effects for all these models were identical and included the scaled count of the fluorescent cells and the dilution effect

Model	Random effects	AICc	Δ AICc	W
B	1. Test date as random intercept and random slope	91.62	0.0	0.71
	2. Test date as random intercept and random slope + number of field as random intercept	93.49	1.87	0.28
	3. Test date as random intercept	102.32	10.70	0.0
	4. Test date as random intercept + number of field as random intercept	104.28	12.66	0.0
L	1. Test date as random intercept and random slope + number of field as random intercept	-1746.59	0.0	0.7
	2. Test date as random intercept and random slope	-1744.89	1.70	0.3
	3. Test date as random intercept + number of field as random intercept	-1637.32	109.27	0.0
	4. Test date as random intercept	-1637.30	109.29	0.0

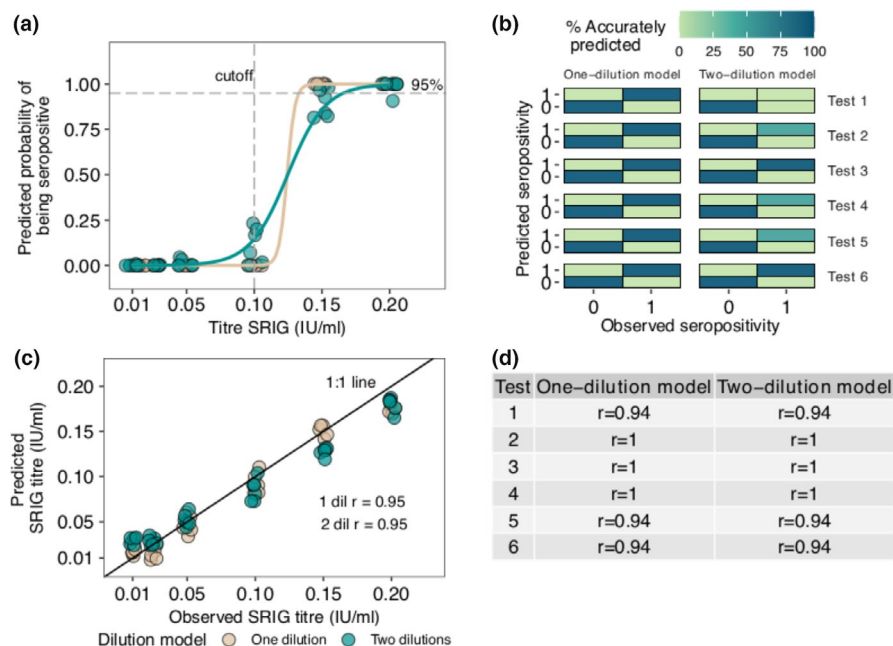


FIGURE 3 Results of the models fit to the SRIG control data. All graphs compare the one-dilution (1:10) and the two-dilution (1:10 and 1:25) models. (a) Model fit of the titres of the SRIG concentration curve versus the predicted probability from the binomial model. The horizontal dash line represents the 95% probability, and the vertical dash line represents the previously set threshold at 0.1 IU/ml. (b) Sensitivity and specificity of the binomial prediction per test date. (c) Correlation of the titres of the observed SRIG values versus the predicted values with the log-normal models. (d) Comparison of the Spearman coefficients between the correlations of the observed titres versus the predicted titres per test date [Colour figure can be viewed at wileyonlinelibrary.com]

the FAVN, using binomial predictions from dog sera. The same data were used to estimate the sensitivity (accurately predicted positive/(accurately predicted positive + inaccurately predicted negative)) and specificity (accurately predicted negative/(accurately predicted negative + inaccurately predicted positive)) of the pmRFFIT. Sera with FAVN titres > 0.1 IU/ml were classified as positive and ≤ 0.1 IU/ml as negative. Only data from the first pmRFFIT test date were used under

the assumption that future applications would preferably use a single test. To compare pmRFFIT accuracy in different FAVN titre ranges, the RVNA values obtained through FAVN were categorized through the *cut* function in R. The number of categories was obtained by dividing the range of the RVNA titre values (maximum minus minimum value obtained through FAVN) by the optimal category width, calculated with the Freedman–Diaconis rule (Freedman & Diaconis, 1981).

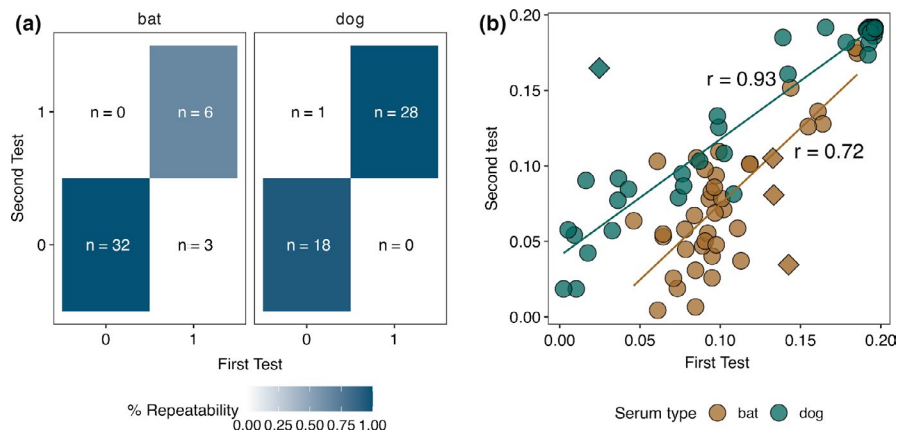


FIGURE 4 Repeatability of the predictions in bat ($N = 41$) and dog ($N = 47$) samples. (a) Percentage of repeatability of the seropositive outcome of the bat and dog serum samples with the pmRFFIT test. (b) Linear correlation of the predicted titres between the first and second tests for dog serum and bat serum. Diamonds indicate samples that were contradictory in the binomial prediction [Colour figure can be viewed at wileyonlinelibrary.com]

We later re-evaluated accuracy for weak positives using only the lowest titre FAVN samples. Since we designed the pmRFFIT to quantify low RVNA titres and many of the available dog sera had RVNA titres above the range of our test, direct comparison of continuous titre predictions for the whole range was not feasible. Instead, we compared predicted pmRFFIT titres using the relevant FAVN range (0–0.22 IU/ml). To evaluate the sensitivity and specificity of different threshold values in the log-normal predictions, we applied a receiver operating characteristic (ROC) curve analysis. The ROC curve was produced across all the range of evaluated titres, using a threshold of > 0.1 IU/ml (positives) as the benchmark reference.

3 | RESULTS

We assessed the variability of the pmRFFIT using the SRIG titre concentration curves generated in 6 different dates ("Test 1" through "Test 6"). As expected, the number of infected cells declined at higher SRIG titres in all replicates; however, the shape of the antibody decay curve varied across test dates (Figure 2). At the 0.1 IU/ml SRIG concentration, infected cell counts were more dispersed in the 1:25 dilution than in the 1:10 dilution, as indicated by higher IQR within each of the 6 test dates. Across all the SRIG concentrations in all test dates ($N = 36$), 77.78% of the count comparisons were less dispersed in the 1:10 dilution suggesting this dilution could be more precise for downstream statistical analysis (SI, Figure S2).

3.1 | Prediction of seropositivity using binomial GLMM

Binomial GLMMs accurately predicted seropositive and seronegative SRIG concentrations (Figure 3). The best random effects for the binomial model included a random slope and intercept for test date (Table 1). The models built with the 1:10 dilution data only ("one-dilution model") and from both the 1:10 and 1:25 dilution data ("two-dilution model") had equivalent specificity (100%), but

the one-dilution model was more sensitive (100% versus 58.33%, Figure 3a,b). Furthermore, the two-dilution binomial model failed to correctly predict the seropositive controls on 4 out of the 6 test dates, confirming improved performance of the one-dilution model (Figure 3b).

3.2 | Prediction of titre values using log-normal GLMM

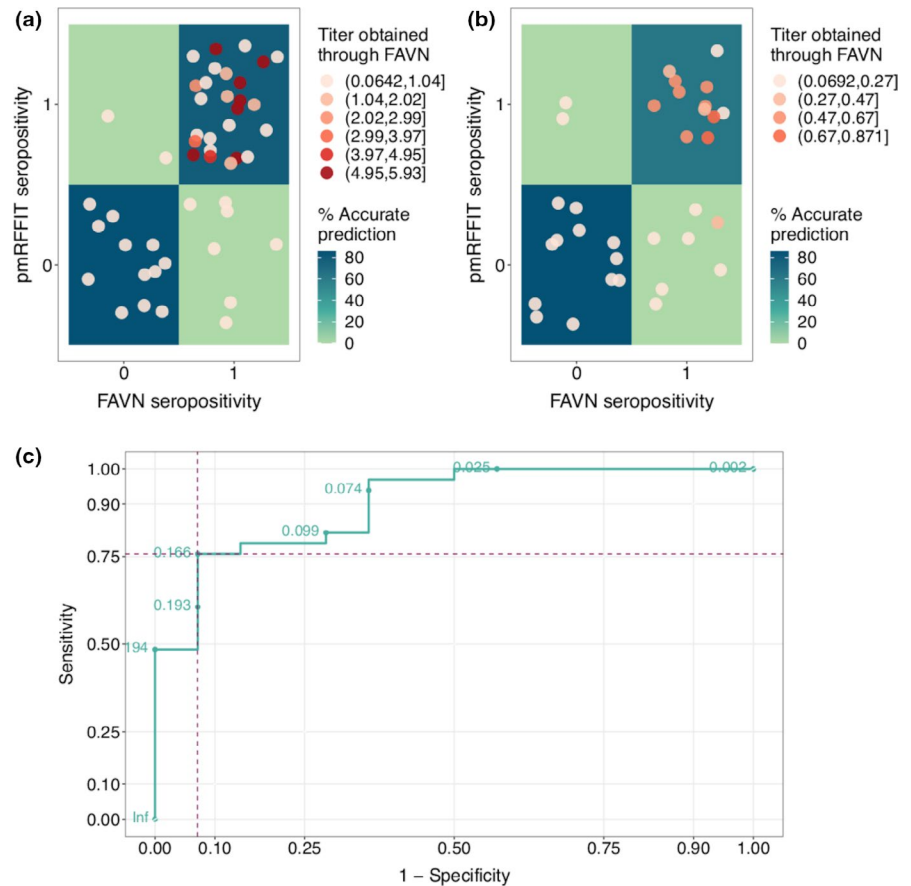
The log-normal GLMMs accurately predicted RVNA titres from the data sets generated through our protocol on different test dates (Figure 3). The best log-normal model included a random intercept and slope for test date. Although the most complex model had the lowest AICc, the simpler model (without the random intercept of field) had a $\Delta AICc < 2$ (Table 1). Observed and predicted SRIG titres were highly correlated for both the one- and two-dilution models ($r = 0.95$, Figure 3c). When comparing test dates (i.e., one-to-one comparison between correlations of the one-dilution and the two-dilution model from the same test dates), the correlation coefficients were similar, suggesting the simpler one-dilution model is sufficient for titre prediction (Figure 3d).

3.3 | Repeatability, accuracy, sensitivity and specificity with animal sera

Among all the animal serum samples ($N = 88$), 95.45% were consistently predicted as seropositive or seronegative (92.68% for bats and 97.87% for dogs). Bat samples ($N = 41$) had a repeatability of 66.7% for positive samples (6/9 positive samples were positive in the second round) and 100% for negative samples ($N = 32$). Dog samples ($N = 47$) had 100% repeatability for positive samples ($N = 28$) and 94.74% for negative samples (18/19 negative samples remained negative in the second round) (Figure 4a, SI, Figure S4). Predicted titres between test repetitions were correlated for both sera (both $r = 0.81$, bat $r = 0.72$, dog $r = 0.93$, Figure 4b).

Relative to FAVN, pmRFFIT predictions using the binomial model on dog sera showed overall moderate accuracy (80.43%

FIGURE 5 Validation of pmRFFIT with the FAVN on the dog serum samples. (a) Seropositivity comparison for all FAVN titres: sensitivity = 78.79%; specificity = 85.71%; overall accuracy = 80.85% ($N = 47$). (b) Seropositive comparison of lower FAVN titres: overall accuracy = 72.73% ($N = 33$). (c) ROC curve of the log-normal titre prediction of the first test compared to the FAVN at threshold > 0.1 IU/ml for seropositivity. The continuous thresholds for the pmRFFIT are in green [Colour figure can be viewed at wileyonlinelibrary.com]



of samples in agreement with FAVN; sensitivity = 78.79%; specificity = 84.62%). Dog samples categorized as positive according to the binomial prediction ($N = 28$) had a higher correct prediction. Only 2 positive samples failed in the pmRFFIT prediction compared to the FAVN. For dog samples categorized as negative by the binomial pmRFFIT ($N = 9$), 7 samples were contradictory. These misclassified samples were consistently predicted as negative in the pmRFFIT, and titres displayed a strong correlation between tests ($r = 0.93$, SI, Table S3). The binomial model was most accurate for samples with high RVNA titres (> 1.04 IU/ml, $N = 13$; 100% predicted to be seropositive; Figure 5a). At lower FAVN titres (< 1.04 IU/ml, $N = 33$), 72.73% of samples were predicted correctly by the pmRFFIT. To evaluate the accuracy of the binomial prediction in this lower range, the titre categorization process was repeated using the subset of data with FAVN titres ≤ 0.871 IU/ml ($N = 33$). Among the higher titre samples in this particular set (> 0.27 to ≤ 0.871 IU/ml, $N = 11$), 90.91% were correctly predicted as seropositive (Figure 5b). From the samples with titres ≤ 0.27 IU/ml, only 63.64% were correctly predicted ($N = 22$, Figure 5b). Accuracy rose to 83.33% in the lowest FAVN titre range (≤ 0.07 IU/ml, $N = 12$). The pmRFFIT titres from the log-normal prediction were correlated with FAVN titres ($r = 0.51$ – 0.85 , depending on the data subset, SI Figure S3). The ROC curve relative to the FAVN titre values showed that threshold values around 0.166 IU/ml maximized sensitivity and specificity.

4 | DISCUSSION

The most commonly applied serological tests to detect RVNA titres challenge a range of serial dilutions of serum with infectious RV. This process is labour-intensive and requires laboratory capacity to grow large quantities of pathogenic RV. Here, we provide an alternative serological framework that uses a combination of digital image analysis and statistical analysis to estimate the presence and titre of RVNA from a single dilution using only 3.5 μ l of serum.

The pmRFFIT differs from other lyssavirus neutralization tests in several key aspects. It uses an MLV(RG) pseudotype rather than pathogenic RV, allowing the pmRFFIT to be performed in any low-containment laboratory with appropriate cell culture and microscopy facilities. The addition of GFP expression is significant, since it removes the need for FITC-conjugated antibody (reducing reagent costs) and the fixation and staining steps used in traditional RFFIT or FAVN. One potential drawback of using GFP expression to measure infectivity is the prolonged neutralization period (66-hr versus 24-hr RFFIT and 48-hr FAVN) required to gain sufficient fluorescence for image processing (Aubert, 1992; Smith et al., 1973). Longer neutralization requirements (60 hr) were also required in a FAVN modification using a GFP expressing recombinant CVS-11-eGFP but did not alter results relative to the test run with CVS-11 (Xue et al., 2014). Fortunately, extended incubations are unlikely to alter neutralization outcomes since MLV(RG) pseudotype is replication incompetent. This prevents infection of additional cells during incubation (Temperton et al., 2015).

The pmRFFIT also uses an imaging pipeline that combines systematic photography of microscope fields with automated digital image processing to count infected cells. Microscopy in neutralization tests is time-consuming and presents challenges for interlaboratory comparisons due to multiple sources of variation, especially those that affect the manual readout (e.g., laboratory user, manual pipetting, uneven cell monolayer) (Briggs et al., 1998; Cliquet et al., 1998; Eschbaumer et al., 2014; Péharpré et al., 1999; Timiryasova et al., 2019). The pmRFFIT standardized approach minimizes these sources of error while potentially reducing microscope operator time. Moreover, the imaging process generates traceable and permanent electronic records of the raw data, eliminating the need to manually digitize records of field counts. Several investigators have previously incorporated image processing into RV neutralization tests. For example, Péharpré et al. (1999) modified the FAVN and RFFIT readout by using an automated image analysis; however, one important drawback was the high cost of the equipment (a motorized stage and the software required for this). The pmRFFIT uses freely available image processing program and plugins (ImageJ) making the approach accessible for any user. Streicker et al. (2012) also used photography and image processing to count pixels using a microRFFIT but did not make full use of the quantitative nature of imaging data to obtain RVNA titres and used pathogenic RV rather than a viral pseudotype. Finally, instead of scoring microscope fields or wells as virus positive or negative across many dilutions to obtain serological status and titre, the pmRFFIT achieves this by quantifying levels of cellular infection in a single serum dilution. The efficacy of this statistical modelling approach highlights the value of historically underutilized quantitative data on cellular infectivity for lyssavirus serology.

Model selection indicated substantial day-to-day variation in the SRIG dilution series. This effect was unsurprising since virus neutralization tests are biologically dynamic systems that can be influenced by many factors (e.g., variability in the humidity of the incubator, technical manipulation, light condition of the microscope, variability in GFP expression in the cells) (Briggs et al., 1998; Hammami et al., 1999; Kostense et al., 2012). Since our statistical approach handles this variability through the random effect of test date, the pmRFFIT is best suited for large numbers of serum samples that require testing to be carried out across multiple batches. However, performance is only marginally reduced when running single models for each test date, implying the pmRFFIT may still be useful when fewer samples are available for testing (see SI, Figures S4 and S5). Surprisingly, fitting the GLMMs to data from a single 1:10 dilution of SRIG predicted both seropositivity and RVNA titre more accurately than models fit to both the 1:10 and 1:25 dilutions. The reduced performance of the two-dilution model reflected higher variability in the 1:25 dilution compared to the 1:10 dilution, as evidenced by greater IQR values (SI, Figure S2). Ultimately, this variability likely reflects both higher stochasticity in infected cell counts at lower serum concentrations and pipetting error. Regardless, the ability to detect low titres (<0.2 IU/ml) with just one dilution and without the need to conduct separate tests for screening and titration is advantageous since manual effort and materials are reduced.

Cell-based experiments are expected to be more variable than other serological diagnostics, and often the allowed variation for test precision is up to 30% (Kostense et al., 2012; Timiryasova et al., 2019). For example, Kostense et al. (2012) evaluated RFFIT repeatability by testing 3 different types of immunoglobulins (HRIG, CL184 and SRIG), and intratest variation was 26%, 18% and 25%, respectively. Similarly, Timiryasova et al. (2019) validated a RFFIT protocol with 15.7% variation among intratest repetitions. In comparison, our test showed higher repeatability for serum samples from dogs. Repeatability was slightly lower in bat samples; however, two out of the 3 conflicting bat samples were explainable as threshold effects. Specifically, predicted titres in both tests occurred near the threshold defining seropositivity and varied by < 0.05 IU/mL between test replicates, indicating only trivial variation between tests (Figure 4b, SI, Table S2). Nevertheless, quantitative titre predictions across replicate runs were less consistent in bat samples relative to dogs (Figure 4b). One explanation for this finding could be linked to serum quality in bat samples arising from the collection of whole blood using peripheral venipuncture and capillary collection, rather than cephalic or jugular venipuncture used for dog samples. Higher haemolysis levels in bat samples could have increased variation in test outcomes (Neumann & Bonistalli, 2009). As a second possibility, predictions of lower titres tended to have a higher variability and titres in bats were on average lower than dogs, many of which were previously vaccinated. Indeed, 26 out of 47 dogs compared to 5 out of 41 bat samples had predicted titres ≥ 0.15 IU/ml. Consistent with our findings, Kostense et al. (2012) also detected that the RFFIT had prediction limitations at lower titre levels (limit of quantification: 0.2 IU/ml, limit of detection: 0.118 IU/ml). Moreover, they observed antibody concentration curves had an initial and evident bend in the titre concentration at 0.2 IU/ml, but the ability to observe slope changes in the lowest concentrations of serum (<0.1 IU/ml) was challenging. Overall, the pmRFFIT was most precise in discriminating strong positives from strong negatives, where the highest and lowest titres were constantly predicted as such. In consideration of these points, standard serological methods might have limitations measuring low RVNA titres (<0.5 IU/ml), while the pmRFFIT grants a highly repeatable approach for these titre values.

Using the external validation set of dog samples with known RVNA titres, the overall accuracy of the pmRFFIT was moderately high (Figure 5). The pmRFFIT was also able to estimate RVNA titres from a single serum dilution which correlated with known FAVN titres (SI Figure S3). Discrepancies occurred in samples with FAVN titres ranging from 0.07 to 0.29 IU/ml but were not observed for samples with higher titres ($N = 23$). In the previous analysis of these samples, sera that did not reach the 0.5 IU/ml threshold were considered negative and were not re-tested (Wright et al., 2009). We therefore cannot disregard the possibility of inaccurate titre assessment through FAVN, considering low-titre detection is even more challenging through classical approaches and that titre prediction through FAVN tests can vary even for higher titres (i.e., samples scoring 0.5 IU/ml can range from 0.38 to 0.66 IU/ml when repeated) (Liu et al., 2012; Wright et al., 2009). Although we did not carry out a formal validation

for the bat samples, the estimated seroprevalence was 0.21 (CI: 0.09, 0.35), consistent with several previous studies using the RFFIT (de Thoisy et al., 2016; Steece & Altenbach, 1989; Streicker et al., 2012, 2013). Nevertheless, the ability of our approach to estimate RVNA titres for all samples tested without additional laboratory work provides an additional layer of information. The titre continuous levels can be further used to gauge confidence in positive or negative predictions. For example, predicted titres abutting the threshold for seropositivity might be viewed with caution.

Selecting a different threshold (i.e., 0.166 from the ROC curve) would have increased the assessment values of the pmRFFIT (e.g., repeatability in bats) but would have predicted low-titre sera as negative. Detection of low RVNA titres is important for epidemiological studies of rabies exposure (Gold et al., 2020). For example, bats sometimes produce low levels of RVNA after viral exposures, and the antibody response can wane to undetectable levels within months after the first exposure (Jackson et al., 2008; Turmelle et al., 2010). Indeed, the predicted titres among our predicted seropositive bat samples were consistently less than < 0.12 IU/ml. For this reason, we designed our test to detect low RVNA titres, with seropositivity defined as > 0.1 IU/ml. The ROC analysis for the dog sera showed this was close to the optimal range of thresholds for sensitivity and specificity. Titres > 0.2 IU/ml, as might be required for studies concerned with protective levels following immunization, cannot currently be estimated accurately. Using pmRFFIT, such samples would be classified as seropositive with a predicted titre of 0.2 IU/ml. If desired, studies aiming to predict RVNA titres higher than the focus of this study would require higher additional serum dilutions in conjunction with a greater range of SRIG concentrations.

In summary, the pmRFFIT quantifies RVNA titres using a single, low-biocontainment test that requires only a single dilution of test sera. We recommend first employing the binomial model at a pre-determined threshold for seropositivity and then using the log-normal model to predict titres for the putatively positive samples (considered positive by the binomial model). If benchmark data are available, it would be possible to select the most sensitive and specific threshold after predicting titres for all available samples using the ROC curve analysis. Downstream analyses could use binomial and continuous data as needed for specific project objectives. The pmRFFIT could be extended to other lyssaviruses for similarly low-biocontainment testing of neutralizing antibodies. The ability to perform the test using the low-volume sera that are often available for studies of wild bats or longitudinal studies of captive bats is a particularly useful feature of our approach. Such studies are increasingly desirable to investigate the transmission dynamics of poorly understood bat viruses.

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ETHICS

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to, and the appropriate ethical review committee approval has been received. Bat capture and sampling methods were approved by the Research Ethics Committee of the University of Glasgow School of Medical Veterinary and Life Sciences (Ref 08a/15).

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study will be openly available in Zenodo. A doi has been reserved for scripts and data at <http://doi.org/10.5281/zenodo.3765714>.

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REFERENCES

- Araujo, D. B., Martorelli, L. A., Kataoka, A. P. G. A., Campos, A. C. A., Rodrigues, C. S., Sanfilippo, L. F., Cunha, E. S., Durigon, E. L., & Favoretto, S. R. (2014). Antibodies to rabies virus in terrestrial wild mammals in native rainforest on the north coast of São Paulo state, Brazil. *Journal of Wildlife Diseases*, 50(3), 469–477. <https://doi.org/10.7589/2013-04-099>
- Aubert, M. (1992). Practical significance of rabies antibodies in cats and dogs. *Revue Scientifique et Technique de l'OIE*, 11(3), 760. <https://doi.org/10.20506/rst.11.3.622>
- Bartoń, K. (2020). MuMIn: Multi-model inference. *R Package Version 1.43.17*. <https://CRAN.R-project.org/package=MuMIn>
- Barton, L. D., & Campbell, J. B. (1988). Measurement of rabies-specific antibodies in carnivores by an enzyme-linked immunosorbent assay. *Journal of Wildlife Diseases*, 24(2), 246–258. <https://doi.org/10.7589/0090-3558-24.2.246>
- Bates, D., Maechler, M., Bolker, B. M., Walker, S. C., & Mächler, M. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, 67(1), 1–48. <https://doi.org/10.18637/jss.v067.i01>

- Becker, D. J., Crowley, D. E., Washburne, A. D., & Plowright, R. K. (2019). Temporal and spatial limitations in global surveillance for bat filoviruses and henipaviruses. *Biology Letters*, 15(12), 1–7. <https://doi.org/10.1098/rsbl.2019.0423>
- Bentley, E. M., Mather, S. T., & Temperton, N. J. (2015). The use of pseudotypes to study viruses, virus sero-epidemiology and vaccination. *Vaccine*, 33(26), 2955–2962. <https://doi.org/10.1016/j.vaccine.2015.04.071>
- Blackwood, J. C., Streicker, D. G., Altizer, S. M., & Rohani, P. (2013). Resolving the roles of immunity, pathogenesis and immigration for rabies persistence in vampire bats. *Proceedings of the National Academy of Sciences*, 110(51), 20837–20842. <https://doi.org/10.1073/pnas.1308817110>
- Bock, M., Bishop, K. N., Towers, G., & Stoye, J. P. (2000). Use of a transient assay for studying the genetic determinants of Fv1 restriction. *Journal of Virology*, 74(16), 7422–7430. <https://doi.org/10.1128/JVI.74.16.7422-7430.2000>
- Borremans, B., Hens, N., Beutels, P., Leirs, H., & Reijnders, J. (2016). Estimating time of infection using prior serological and individual information can greatly improve incidence estimation of human and wildlife infections. *PLOS Computational Biology*, 12(5), e1004882. <https://doi.org/10.1371/journal.pcbi.1004882>
- Briggs, D. J., Smith, J. S., Mueller, F. L., Schwenke, J., Davis, R. D., Gordon, C. R., Schweitzer, K., Orciari, L. A., Yager, P. A., & Rupprecht, C. E. (1998). A comparison of two serological methods for detecting the immune response after rabies vaccination in dogs and cats being exported to rabies-free areas. *Biologicals*, 26(4), 347–355. <https://doi.org/10.1006/biol.1998.0162>
- Campos, A. A. S., Santos, R. N., Benavides, J. A., Carvalho Ruthner Batista, H. B., Finoketti, F., Wagner, P. G. C., Zafalon-Silva, B., Alievi, M., Silva, F. B., Witt, A., Tartarotti, A., Cassia Rodrigues da Silva, A., Ferreira, K. C. S., Frazzon, A. P. G., Roehe, P. M., & Franco, A. C. (2019). Rabies surveillance in wild mammals in South Of Brazil. *Transboundary and Emerging Diseases*, October, 1–8. <https://doi.org/10.1111/tbed.13415>
- Cliquet, F., Aubert, M., & Sagné, L. (1998). Development of a fluorescent antibody virus neutralisation test (FAVN test) for the quantitation of rabies-neutralising antibody. *Journal of Immunological Methods*, 212(1), 79–87. [https://doi.org/10.1016/S0022-1759\(97\)00212-3](https://doi.org/10.1016/S0022-1759(97)00212-3)
- Cliquet, F., McElhinney, L. M., Servat, A., Boucher, J. M., Lowings, J. P., Goddard, T., Mansfield, K. L., & Fooks, A. R. (2004). Development of a qualitative indirect ELISA for the measurement of rabies virus-specific antibodies from vaccinated dogs and cats. *Journal of Virological Methods*, 117(1), 1–8. <https://doi.org/10.1016/j.jviro.2003.12.001>
- Condit, R. C. (2001). Principles of virology. In D. M. Knipe & P. M. Howley (Eds.), *Fields Virology* 4th edn. (Vol. 1, 19–51). Philadelphia: Lippincott Williams & Wilkins.
- Constantine, D. G., Tierkel, E. S., Kleckner, M. D., & Hawkins, D. M. (1968). Rabies in New Mexico cavern bats. *Public Health Reports (Washington, DC: 1896)*, 83(4), 303–316. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1891046/pdf/pubhealthreporig00004-0041.pdf>
- Costa, L. J. C., Andrade, F. A. G., Uieda, W., Martorelli, L. F. A., Kataoka, A. P. A. G., & Fernandes, M. E. B. (2013). Serological investigation of rabies virus neutralizing antibodies in bats captured in the eastern Brazilian Amazon. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 107(11), 684–689. <https://doi.org/10.1093/trstmh/trt080>
- Cunningham, A. A., Daszak, P., & Wood, J. L. N. (2017). One Health, emerging infectious diseases and wildlife: Two decades of progress? *Philosophical Transactions of the Royal Society B: Biological Sciences*, 372(1725), 20160167. <https://doi.org/10.1098/rstb.2016.0167>
- Daszak, P., Cunningham, A. A., & Hyatt, A. D. (2000). Emerging infectious diseases of wildlife - threats to biodiversity and human health. *Science*, 287(5459), 443–449. <https://doi.org/10.1126/science.287.5452.443>
- De Benedictis, P., Mancin, M., Cattoli, G., Capua, I., & Terrregino, C. (2012). Serological methods used for rabies post vaccination surveys: An analysis. *Vaccine*, 123(7–8), 278–285. <https://doi.org/10.1016/j.vaccine.2012.06.089>
- Marcelo Azevedo de Paula Antunes, J., de Castro Demoner, L., Morosini de Andrade Cruvinel, T., Paula Kataoka, A., Fátima Alves Martorelli, L., Puglia Machado, G., & Megid, J. (2017). Rabies virus exposure of Brazilian free-ranging wildlife from municipalities without clinical cases in humans or in terrestrial wildlife. *Journal of Wildlife Diseases*, 53(3), 662–666. <https://doi.org/10.7589/2016-09-204>
- de Thoisy, B., Bourhy, H., Delaval, M., Pontier, D., Dacheux, L., Darcissac, E., Donato, D., Guidez, A., Larrous, F., Lavenir, R., Salmier, A., Lacoste, V., & Lavergne, A. (2016). Bioecological drivers of rabies virus circulation in a neotropical bat community. *PLoS Neglected Tropical Diseases*, 10(1), 1–21. <https://doi.org/10.1371/journal.pntd.0004378>
- Eschbaumer, M., Law, S., Solis, C., Chernick, A., van der Meer, F., & Czub, M. (2014). Rapid detection of neutralizing antibodies against bovine viral diarrhoea virus using quantitative high-content screening. *Journal of Virological Methods*, 198, 56–63. <https://doi.org/10.1016/j.jviro.2013.12.017>
- Freedman, D., & Diaconis, P. (1981). On the histogram as a density estimator: L2 theory. *Zeitschrift Für Wahrscheinlichkeitstheorie Und Verwandte Gebiete*, 57(4), 453–476. <https://doi.org/10.1007/BF01025868>
- Gamble, A., Garnier, R., Chambert, T., Gimenez, O., & Boulinier, T. (2020). Next-generation serology: Integrating cross-sectional and capture-recapture approaches to infer disease dynamics. *Ecology*, 101(2), <https://doi.org/10.1002/ecy.2923>
- George, D. B., Webb, C. T., Farnsworth, M. L., O'Shea, T. J., Bowen, R. A., Smith, D. L., Stanley, T. R., Ellison, L. E., & Rupprecht, C. E. (2011). Host and viral ecology determine bat rabies seasonality and maintenance. *Proceedings of the National Academy of Sciences*, 108(25), 10208–10213. <https://doi.org/10.1073/pnas.1010875108>
- Gilbert, A. T., Fooks, A. R., Hayman, D. T. S., Horton, D. L., Müller, T., Plowright, R., Peel, A. J., Bowen, R., Wood, J. L. N., Mills, J., Cunningham, A. A., & Rupprecht, C. E. (2013). Deciphering serology to understand the ecology of infectious diseases in wildlife. *EcoHealth*, 10(3), 298–313. <https://doi.org/10.1007/s10393-013-0856-0>
- Gold, S., Donnelly, C. A., Nouvellet, P., & Woodroffe, R. (2020). Rabies virus-neutralising antibodies in healthy, unvaccinated individuals: What do they mean for rabies epidemiology? *PLoS Neglected Tropical Diseases*, 14(2), e0007933. <https://doi.org/10.1371/journal.pntd.0007933>
- Goodin, D. G., Jonsson, C. B., Allen, L. J. S., & Owen, R. D. (2018). Integrating landscape hierarchies in the discovery and modeling of ecological drivers of zoonotically transmitted disease from wildlife. In *The Connections Between Ecology and Infectious Disease, Advances in Environmental Microbiology*, 5, 299–317. Tx. https://doi.org/10.1007/978-3-319-92373-4_9
- Gurley, E. S., Hegde, S. T., Hossain, K., Sazzad, H. M. S., Hossain, M. J., Rahman, M., Sharker, M. A. Y., Salje, H., Islam, M. S., Epstein, J. H., Khan, S. U., Kilpatrick, A. M., Daszak, P., & Luby, S. P. (2017). Convergence of humans, bats, trees, and culture in Nipah virus transmission, Bangladesh. *Emerging Infectious Diseases*, 23(9), 1446–1453. <https://doi.org/10.3201/eid2309.161922>
- Hammami, S., Schumacher, C., Cliquet, F., Tlatli, A., Aubert, A., & Aubert, M. (1999). Vaccination of Tunisian dogs with the lyophilised SAG2 oral rabies vaccine incorporated into the DBL2 dog bait. *Veterinary Research*, 30(6), 607–613. <https://doi.org/hal-00902600>
- Hierholzer, J. C., & Killington, R. A. (1996). Virus isolation and quantitation. In Brian W. J. Mahy & Hillar O. Kangro (Eds.), *Virology Methods Manual* (pp. 25–46). London: Academic Press. <https://doi.org/10.1016/B978-012465330-6/50003-8>
- Horton, D. L., Breed, A. C., Arnold, M. E., Smith, G. C., Aegerter, J. N., McElhinney, L. M., Johnson, N., Banyard, A. C., Raynor, R., Mackie,

- I., Denwood, M. J., Mellor, D. J., Swift, S., Racey, P. A., & Fooks, A. R. (2020). Between roost contact is essential for maintenance of European bat lyssavirus type-2 in *Myotis daubentonii* bat reservoir: 'The Swarming Hypothesis'. *Scientific Reports*, 10(1), 1740. <https://doi.org/10.1038/s41598-020-58521-6>
- Irie, T., & Kawai, A. (2002). Studies on the different conditions for rabies virus neutralization by monoclonal antibodies #1-46-12 and #7-1-9. *Journal of General Virology*, 83(12), 3045-3053. <https://doi.org/10.1099/0022-1317-83-12-3045>
- Jackson, F. R., Turmelle, A. S., Farino, D. M., Franka, R., McCracken, G. F., & Rupprecht, C. E. (2008). Experimental rabies virus infection of big brown bats (*Eptesicus fuscus*). *Journal of Wildlife Diseases*, 44(3), 612-621. <https://doi.org/10.7589/0090-3558-44.3.612>
- Jorge, R. S. P., Pereira, M. S., Morato, R. G., Scheffer, K. C., Carnieli, P., Ferreira, F., Furtado, M. M., Kashivakura, C. K., Silveira, L., Jacomo, A. T. A., Lima, E. S., de Paula, R. C., & May-Junior, J. A. (2010). Detection of rabies virus antibodies in Brazilian free-ranging wild carnivores. *Journal of Wildlife Diseases*, 46(4), 1310-1315. <https://doi.org/10.7589/0090-3558-46.4.1310>
- Kerins, J., Koske, S., Kazmierczak, J., Austin, C., Gowdy, K., & Dibbernardo, A. (2018). Outbreak of Seoul virus among rats and rat owners—United States and Canada, 2017. *Canada Communicable Disease Report*, 44(2), 71-74. <https://doi.org/10.14745/ccdr.v44i02a07>
- Kostense, S., Moore, S., Companjen, A., Bakker, A. B. H., Marissen, W. E., von Eyben, R., Weverling, G. J., Hanlon, C., & Goudsmit, J. (2012). Validation of the rapid fluorescent focus inhibition test for rabies virus-neutralizing antibodies in clinical samples. *Antimicrobial Agents and Chemotherapy*, 56(7), 3524-3530. <https://doi.org/10.1128/AAC.06179-11>
- Kuzmin, I. V., Niezgoda, M., Franka, R., Agwanda, B., Markotter, W., Beagley, J. C., Urazova, O. Y., Breiman, R. F., & Rupprecht, C. E. (2008). Lagos bat virus in Kenya. *Journal of Clinical Microbiology*, 46(4), 1451-1461. <https://doi.org/10.1128/JCM.00016-08>
- Liu, Y., Zhang, S., Zhang, F., & Hu, R. (2012). A semi-quantitative serological method to assess the potency of inactivated rabies vaccine for veterinary use. *Virologica Sinica*, 27(4), 259-264. <https://doi.org/10.1007/s12250-012-3260-y>
- Ma, X., Monroe, B. P., Cleaton, J. M., Orciari, L. A., Gigante, C. M., Kirby, J. D., & Wallace, R. M. (2020). Rabies surveillance in the United States during 2018. *Journal of the American Veterinary Medical Association*, 256(2), 195-208. <https://doi.org/10.2460/javma.256.2.195>
- Ma, X., Niezgoda, M., Blanton, J. D., Recuenco, S. E., & Rupprecht, C. E. (2012). Evaluation of a new serological technique for detecting rabies virus antibodies following vaccination. *Vaccine*, 30, 5358-5362. <https://doi.org/10.1016/j.vaccine.2012.06.037>
- Mather, S. T., Scott, S. D., Temperton, N. J., Wright, E., King, B., & Daly, J. (2013). Current progress with serological assays for exotic emerging/re-emerging viruses. *Future Virology*, 8(8), 745-755. <https://doi.org/10.2217/fvl.13.60>
- Mena, I., Nelson, M. I., Quezada-Monroy, F., Dutta, J., Cortes-Fernández, R., Lara-Puente, J. H., Castro-Peralta, F., Cunha, L. F., Trovão, N. S., Lozano-Dubernard, B., Rambaut, A., van Bakel, H., & García-Sastre, A. (2016). Origins of the 2009 H1N1 influenza pandemic in swine in Mexico. *Elife*, 5(JUN2016). <https://doi.org/10.7554/eLife.16777>
- Metcalfe, C. J. E., Farrar, J., Cutts, F. T., Basta, N. E., Graham, A. L., Lessler, J., Ferguson, N. M., Burke, D. S., & Grenfell, B. T. (2016). Use of serological surveys to generate key insights into the changing global landscape of infectious disease. *The Lancet*, 388(10045), 728-730. [https://doi.org/10.1016/S0140-6736\(16\)30164-7](https://doi.org/10.1016/S0140-6736(16)30164-7)
- Moeschler, S., Locher, S., Conzelmann, K.-K., Krämer, B., & Zimmer, G. (2016). Quantification of lyssavirus-neutralizing antibodies using vesicular stomatitis virus pseudotype particles. *Viruses*, 8(9), 254. <https://doi.org/10.3390/v8090254>
- Moore, S. M., & Hanlon, C. A. (2010). Rabies-specific antibodies: Measuring surrogates of protection against a fatal disease. *PLoS Neglected Tropical Diseases*, 4(3), e595. <https://doi.org/10.1371/journal.pntd.0000595>
- Neumann, E. J., & Bonistalli, K. N. (2009). Effect of blood sample handling post-collection on *Erysipelothrix rhusiopathiae* antibody titres. *Veterinary Journal*, 180(3), 325-329. <https://doi.org/10.1016/j.tvjl.2008.07.020>
- Obregón-Morales, C., Aguilar-Setién, Á., Perea Martínez, L., Galvez-Romero, G., Martínez-Martínez, F. O., & Aréchiga-Ceballos, N. (2017). Experimental infection of *Artibeus intermedius* with a vampire bat rabies virus. *Comparative Immunology, Microbiology and Infectious Diseases*, 52, 43-47. <https://doi.org/10.1016/J.CIMID.2017.05.008>
- Péharpré, D., Cliquet, F., Sagné, E., Renders, C., Costy, F., & Aubert, M. (1999). Comparison of visual microscopic and computer-automated fluorescence detection of rabies virus neutralizing antibodies. *Journal of Veterinary Diagnostic Investigation*, 11(4), 330-333. <https://doi.org/10.1177/104063879901100406>
- Plowright, R. K., Becker, D. J., McCallum, H., & Manlove, K. R. (2019). Sampling to elucidate the dynamics of infections in reservoir hosts. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 374(1782), 20180336. <https://doi.org/10.1098/rstb.2018.0336>
- Plowright, R. K., Peel, A. J., Streicker, D. G., Gilbert, A. T., McCallum, H., Wood, J., Baker, M. L., & Restif, O. (2016). Transmission or within-host dynamics driving pulses of zoonotic viruses in reservoir-host populations. *PLOS Neglected Tropical Diseases*, 10(8), e0004796. <https://doi.org/10.1371/journal.pntd.0004796>
- Python Core Team. (2019). *Python: A dynamic, open source programming language*. <https://www.python.org/>
- R Core Team. (2020). *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing. <https://www.r-project.org/>
- Rasband, W. S. (2018). *ImageJ*. National Institutes of Health. <https://imagej.nih.gov/ij/>
- Reynes, J.-M., Molia, S., Audry, L., Hout, S., Ngin, S., Walston, J., & Bourhy, H. (2004). Serologic evidence of lyssavirus infection in bats, Cambodia. *Emerging Infectious Diseases*, 10(12), 2231-2234. <https://doi.org/10.3201/eid1012.040459>
- Rupprecht, C. E., Kuzmin, I. V., & Meslin, F. (2017). Lyssaviruses and rabies: Current conundrums, concerns, contradictions and controversies. *F1000Research*, 6, 184. <https://doi.org/10.12688/f1000research.10416.1>
- Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.-Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P., & Cardona, A. (2012, July 28). Fiji: An open-source platform for biological-image analysis. *Nature Methods*, 9(7), 676-682. <https://doi.org/10.1038/nmeth.2019>
- Schneider, M. C., Romijn, P. C., Uieda, W., Tamayo, H., Silva, D. F. D., Belotto, A., Silva, J. B. D., & Leanes, L. F. (2009). Rabies transmitted by vampire bats to humans: An emerging zoonotic disease in Latin America? *Revista Panamericana De Salud Pública*, 25(3), 260-269. <https://doi.org/10.1590/S1020-49892009000300010>
- Smith, J. S., Yager, P. A., & Baer, G. M. (1973). A rapid reproducible test for determining rabies neutralizing antibody. *Bulletin of the World Health Organization*, 48(5), 535-541. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2482941/pdf/bullwho00178-0027.pdf>
- Steece, R., & Altenbach, J. S. (1989). Prevalence of rabies specific antibodies in the Mexican free-tailed bat (*Tadarida brasiliensis mexicana*) at lava cave, New Mexico. *Journal of Wildlife Diseases*, 25(4), 490-496. <https://doi.org/10.7589/0090-3558-25.4.490>
- Streicker, D. G., Franka, R., Jackson, F. R., & Rupprecht, C. E. (2013). Anthropogenic roost switching and rabies virus dynamics in house-roosting big brown bats. *Vector-Borne and Zoonotic Diseases*, 13(7), 498-504. <https://doi.org/10.1089/vbz.2012.1113>
- Streicker, D. G., Recuenco, S., Valderrama, W., Gomez Benavides, J., Vargas, I., Pacheco, V., Condori Condori, R. E., Montgomery, J.,

- Rupprecht, C. E., Rohani, P., & Altizer, S. (2012). Ecological and anthropogenic drivers of rabies exposure in vampire bats: Implications for transmission and control. *Proceedings of the Royal Society B: Biological Sciences*, 279(1742), 3384–3392. <https://doi.org/10.1098/rspb.2012.0538>
- Temperton, N. J., Wright, E., & Scott, S. D. (2015). Retroviral pseudotypes - From scientific tools to clinical utility. In *eLS* (pp. 1–11). : John Wiley & Sons, Ltd. <https://doi.org/10.1002/9780470015902.a0021549.pub2>.
- Timiryasova, T. M., Luo, P., Zheng, L., Singer, A., Zedar, R., Garg, S., Petit, C., Moore, S., Hu, B. T., & Brown, M. (2019). Rapid fluorescent focus inhibition test optimization and validation: Improved detection of neutralizing antibodies to rabies virus. *Journal of Immunological Methods*, 474, 112626. <https://doi.org/10.1016/j.jim.2019.06.017>
- Towers, G., Bock, M., Martin, S., Takeuchi, Y., Stoye, J. P., & Danos, O. (2000). A conserved mechanism of retrovirus restriction in mammals. *Proceedings of the National Academy of Sciences*, 97(22), 12295–12299. <https://doi.org/10.1073/pnas.200286297>
- Turmelle, A. S., Jackson, F. R., Green, D., McCracken, G. F., & Rupprecht, C. E. (2010). Host immunity to repeated rabies virus infection in big brown bats. *Journal of General Virology*, 91(9), 2360–2366. <https://doi.org/10.1099/vir.0.020073-0>
- Wasniewski, M., & Cliquet, F. (2012). Evaluation of ELISA for detection of rabies antibodies in domestic carnivores. *Journal of Virological Methods*, 179(1), 166–175. <https://doi.org/10.1016/j.jviro.2011.10.019>
- Welch, R. J., Anderson, B. L., & Litwin, C. M. (2009). An evaluation of two commercially available ELISAs and one in-house reference laboratory ELISA for the determination of human anti-rabies virus antibodies. *Journal of Medical Microbiology*, 58(6), 806–810. <https://doi.org/10.1099/jmm.0.006064-0>
- WHO. (2017). Human rabies: 2016 updates and call for data. *Weekly epidemiological record*, 92(7), 77–88. <https://www.who.int/wer/2017/wer9207/en/>
- WHO. (2018). *WHO expert consultation on rabies, third report*, WHO Technical Report Series, No. 1012. (pp. 1–183). Geneva: World Health Organization.. World Health Organization technical report series. Retrieved from <http://www.who.int/bookorders>
- Wright, E., Hayman, D. T. S., Vaughan, A., Temperton, N. J., Wood, J. L. N., Cunningham, A. A., Suu-Ire, R., Weiss, R. A., & Fooks, A. R. (2010). Virus neutralising activity of African fruit bat (*Eidolon helvum*) sera against emerging lyssaviruses. *Virology*, 408(2), 183–189. <https://doi.org/10.1016/j.virol.2010.09.014>
- Wright, E., McNabb, S., Goddard, T., Horton, D. L., Lembo, T., Nel, L. H., Weiss, R. A., Cleaveland, S., & Fooks, A. R. (2009). A robust lentiviral pseudotype neutralisation assay for in-field serosurveillance of rabies and lyssaviruses in Africa. *Vaccine*, 27, 7178–7186. <https://doi.org/10.1016/j.vaccine.2009.09.024>
- Wright, E., Temperton, N. J., Marston, D. A., McElhinney, L. M., Fooks, A. R., & Weiss, R. A. (2008). Investigating antibody neutralization of lyssaviruses using lentiviral pseudotypes: A cross-species comparison. *Journal of General Virology*, 89(9), 2204–2213. <https://doi.org/10.1099/vir.0.2008/000349-0>
- Xue, X., Zheng, X., Liang, H., Feng, N. A., Zhao, Y., Gao, Y., Wang, H., Yang, S., & Xia, X. (2014). Generation of recombinant rabies virus CVS-11 expressing eGFP applied to the rapid virus neutralization test. *Viruses*, 6(4), 1578–1589. <https://doi.org/10.3390/v6041578>
- Zhou, P., Yang, X.-L., Wang, X.-G., Hu, B., Zhang, L., Zhang, W., & Shi, Z.-L. (2020). A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature*, 579(7798), 270–273. <https://doi.org/10.1038/s41586-020-2012-7>
- Zuur, A. F., Ieno, E. N., Walker, N. J., Saveliev, A. A., & Smith, G. M. (2009). Mixed effects modelling for nested data. In A. F. Zuur E. N. Ieno N. J. Walker A. A. Saveliev & G. M. Smith (Eds.), *Mixed effects models and extensions in ecology with R* (pp. 101–142). New York, NY: Springer. https://doi.org/10.1007/978-0-387-87458-6_5

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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